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Review

# Antiviral effects of human microRNAs and conservation of their target sites

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ABSTRACT

**MicroRNAs are small non-coding RNAs that modulate gene expression at post-transcriptional level, playing a crucial role in cell differentiation and development. Recently, some reports have shown that a limited number of mammalian microRNAs also display antiviral effects. This article summarizes the data in the field paying a special attention to the conservation of the microRNA target sequences in the viral populations. This issue is relevant both for the evaluation of the biological significance of the antiviral effects and for the development of microRNA-based strategies for antiviral intervention.**



## 1. MicroRNA biogenesis and mechanism of action

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene expression at post-transcriptional level by targeting mRNAs for degradation or by inhibiting translation [1]. At the time of writing, the miRNA database (http://www.mirbase.org) [2] contained ~1100 human miRNAs which may regulate up to 30% of the protein-coding genes [3]. MicroRNAs have been shown to play a prevalent role in development and cell differentiation, maintenance of stem cell character, and apoptosis [4,5]. Moreover, several studies indicate that miRNA genes may act as oncogenes or tumor suppressors [6,7].

MicroRNAs are generally transcribed by RNA polymerase II as long precursors, pri-miRNAs, which are processed in the nucleus to ~70 nt hairpin structures by the enzyme Drosha. These products, pre-miRNAs, are then transported to the cytoplasm and processed into ~22 nt miRNA duplexes by the action of the multi-domain RNase III-like enzyme Dicer [8]. Each miRNA duplex is then unwound and the strand with the lower stability in the 5′ end (guide strand) is preferentially selected [9] and incorporated into a large, dynamic multi-protein complex called RNA-induced silencing complex (RISC). The targets of miRNA-loaded RISC are mRNAs presenting a near-perfect sequence complementarity with nucleotides 2–7 in the 5′ portion of the miRNA (the so-called seed region), and additional base pairings with its 3′ region. RISC mediates down-regulation of target gene expression by cleavage or translational inhibition of the target mRNA. The choice between these two modes of action is dictated by the degree of complementarity between miRNA and its target. Near-perfect complementarity produces cleavage of the mRNA, followed by its complete degradation, whereas partial complementarity causes translational inhibition [10]. In animals, endonucleolytic cleavage is rare. Most of the times, miRNA binding to mRNA leads to inhibition of translation or mRNA de-adenylation and subsequent exonucleolytic cleavage [11].

RNA-mediated post-transcriptional gene silencing can also be triggered by exogenous dsRNA molecules. In plants and invertebrates, viral dsRNA molecules are processed into small interfering RNAs (siRNAs) by Dicer and are incorporated into RISC to target pathogen's genome and mRNAs for cleavage [12,13]. Although undisputed in plants and invertebrates, a defensive role for RNA silencing in vertebrates has not been sufficiently defined. In vertebrates, cell exposure to long dsRNA triggers the interferon response as primary form of innate antiviral defense. This leads to a global shutdown in protein translation, cellular RNA degradation and often the death of virus-infected cells [14]. Nevertheless, recent data provide increasing evidence that vertebrate miRNAs can directly affect viral gene expression.

## 2. Primate foamy virus type 1

Primate foamy virus type 1 (PFV-1) is a retrovirus causing persistent infections in non-human primates. PFV-1 can sporadically

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be transmitted to humans where infection is generally asymptomatic. In 2005 Lecellier et al. found that the human miRNA hsa-miR-32 can target the open reading frame 2 of PFV-1, shared by Bet and EnvBet proteins (Fig. 1), thus inhibiting viral translation and reducing the accumulation of the retrovirus in cultured Hela and BHK21 cells [15,16]. In the same study, Tas protein of PFV-1 was shown to suppress microRNA-mediated functions in mammalian cells, counteracting the effect of hsa-miR-32. These results indicated that RNA silencing limits the replication of PFV-1 and that miR-32 contribute substantially to this effect. Overall this work represented the first report of a human miRNA with a direct antiviral effect. It will also be interesting to test the expression level of miR-32 in salivary glands, where PFV normally establishes infection.

## 3. Hepatitis C virus

Hepatitis C virus (HCV) is a member of the Flaviviridiae family with a single-stranded RNA genome of positive polarity, whose replication is assisted by a virally encoded RNA-dependent RNA polymerase. HCV infection is one of the major causes of chronic liver disease, including cirrhosis and liver cancer. In 2007 Pedersen et al. found that interferon β upregulates cellular expression of hsa-miR-196 and hsa-miR-448 and that these miRNAs inhibit HCV replication in cultured hepatic cells [17]. MiR-196 was shown to target the non-structural protein 5A (NS5A) coding sequence whereas miR-448 had its target site in the Core protein ORF (Fig. 1). Transfection of synthetic mimics of miR-196 and miR-448 reproduced the effect of interferon β on HCV replication whereas their neutralization by miRNA-inhibitors reduced the antiviral effect of interferon against HCV. The anti-HCV activity of miR-196 was later confirmed by Hou et al. [18] that showed a marked repression of HCV RNA and protein expression in cultured cells treated with the miR-196 mimic. Finally, another study showed that cellular miR-199a-3p can target the 5′-UTR of HCV genomic RNA, thereby inhibiting viral replication in cultured cells [19].

Overall, these data indicate that three microRNAs expressed in human liver can counteract HCV replication, with two of them

| Human miRNA | virus | target | miRNA-mRNA pairing |
|---|---|---|---|
| 28 | HIV-1 | 3' end of HIV-1 RNA [25] | miRNA: 3'GAGUUAUCUGACACUCGAGGAA5'<br>mRNA: 5'AUCUG-AGCCUGGGAGCUCUC-3' |
| 29a | HIV-1 | Nef protein coding sequence; 3'-UTR of genomic RNA [26, 27] | miRNA: 3'UUGGCU-AAAGUCUACCACGAU5'<br>mRNA: 5'CACUGACCUUUGGAUGGUGCUA3' |
| 32 | PFV-1 | ORF 2 shared by Bet and EnvBet proteins [15] | miRNA: 3'CGUUGAAUCAUUACACGUUAU5'<br>mRNA: 5'GCAGCUU-------UGCAAUA3' |
| 125a-5p | HBV | Overlapping surface antigen and polymerase ORFs [40] | miRNA: 3′AGUGUCCAAUUUC-----CCAGAGUCCCU5′<br>mRNA: 5′UUUUGGGGUGGAGCCCUCAGGCUCAGGGC3′ |
| 125b | HIV-1 | 3' end of HIV-1 RNA [25] | miRNA: 3'AGUGUUCAAU----------CCCAGAGUCCCU5'<br>mRNA: 5'--ACGAGGAUUGUGGAACUUCUGGGACGCAGGGG3' |
| 125b | HPV | Capsid protein L2 coding sequence [36] | miRNA: 3'AGUGUUCA-AUC-CCAG-AGUCCCU5'<br>mRNA: 5'GCACAGGCCUAGAGGUGGGCAGGGA3' |
| 150 | HIV-1 | 3' end of HIV-1 RNA [25] | miRNA: 3'GUGACCA--UGUUCCCAACCCUCU5'<br>mRNA: 5'-UCUGGGACGCAGGGGGUGGGAA-3' |
| 196 | HCV | Nonstructural protein 5A coding sequence [17, 18] | miRNA: 3'GGGUUGUUGUACU----------UUGAUGGAU5'<br>mRNA: 5'ACUGACAAUCUGAAAAUUCCUUGCCAACUACCUU3' |
| 199a-3p | HCV | 5'-UTR of genomic RNA [19] | miRNA: 3'UUGGUUACACGUC-UGAUGACA5'<br>mRNA: 5'CUCCCCUGUGAGGAACUACUGU3' |
| 199a-3p | HBV | Overlapping surface antigen and polymerase ORFs [41] | miRNA: 3'UUGGUUACACGUCUGAUGACA5'<br>mRNA: 5'UUCC--UCUGCAUCCUGCUGC3' |
| 210 | HBV | Overlapping surface antigen and polymerase ORFs [41] | miRNA: 3'AGUCGGCGA-CAGUGUGCGUGUC5'<br>mRNA: 5'AGGACUACUGGCCGGACGCAUGG3' |
| 223 | HIV-1 | 3' end of HIV-1 RNA [25] | miRNA: 3'ACCCCAUAAACUGUU--UGACUGU5'<br>mRNA: 5'AGGGGUCA--GAUAUCCACUGACC3' |
| 382 | HIV-1 | 3' end of HIV-1 RNA [25] | miRNA: 3'GCUUAGGUGGUGCUUGUUGAAG5'<br>mRNA: 5'CGAGCUUGCUACAAGGGACUUU3' |
| 448 | HCV | Core protein coding sequence [17] | miRNA: 3'UACCCUGUA-GGAUGUAUACGUU5'<br>mRNA: 5'GGAGGACGGGGUUAAUUAUGCAA3' |

**Fig. 1.** Human microRNAs with antiviral effects. The miRNA seed sequences are underlined.

being part of the interferon β response. On the other hand, a different study has shown that hsa-miR-122 has an opposite effect on HCV replication. This microRNA is highly expressed in human liver and interacts with the 5′-UTR of HCV genome resulting in up-regulation of viral RNA levels [20]. The mechanism of action of this miRNA has been inquired extensively and reviewed elsewhere [21–23].

## 4. Human immunodeficiency virus type 1

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus causing acquired immunodeficiency syndrome (AIDS), a progressive failure of the immune system leading to life-threatening opportunistic infections and cancers [24]. HIV-1 mainly infects $CD4^+$ T cells, macrophages and dendritic cells. In 2007 Zhang et al. showed that human microRNAs miR-28, miR-125b, miR-150, miR-223, and miR-382 are overexpressed in resting $CD4^+$ T cells and are able to target the 3′ end of human HIV-1 RNA, thus silencing almost all viral messengers [25] (Fig. 1). Specific inhibitors of these cellular miRNAs reversed their effects, measured either as HIV-1 translation or HIV-1 virus production. These data strongly argued for a role of these cellular miRNAs in counteracting viral replication and contributing to HIV latency. Later, Ahluwalia et al. showed that human microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication [26] (Fig. 1). The same microRNA was later found to possess a target site in the 3′-UTR of HIV-1. Inhibiting miR-29a enhanced HIV-1 viral production and infectivity, whereas ectopic expression of a miR-29a mimic suppressed viral replication [27]. These data clearly indicate that several cellular microRNAs can target directly HIV-1 gene expression thereby repressing viral replication. To counteract this cellular defense, HIV-1 has evolved in its Tat protein a suppressor of RNA silencing function. Tat abrogates the cellular RNA-silencing defense by subverting the ability of Dicer to process precursor double-stranded RNAs [28–31]. Also HIV-1 TAR RNA can attenuate RNA-silencing in human cells; it binds to cellular TRBP, an essential partner for Dicer and RISC function [32]. Other viruses, such as Ebola virus and influenza A virus, encode RNA silencing suppressors that are functionally equivalent to HIV-1 Tat [33,34]. Interestingly, there are some data suggesting that cellular microRNAs may counteract influenza A virus infection [35].

## 5. Human papillomavirus

Human papillomavirus (HPV) is a sexually transmitted double-stranded DNA virus whose infection is a cause of cervical intraepithelial neoplasia and cancer. Very recently, Nuovo et al. have shown that hsa-miR-125b may target HPV replication [36]. In situ analysis of human cervical tissue showed a dramatic reduction in hsa-miR-125b expression in the koilocytes, the cytologic marker of productive HPV infection, whereas the expression of the miR-125b precursor was unaffected. Later, cotransfection of NIH 3T3 fibroblasts with HPV genome and hsa-miR-125b mimic or anti-sense inhibitor showed a reduction or increase of viral DNA synthesis, respectively. Sequence analysis of the viral genome revealed a putative binding site for hsa-miR-125b within the coding sequence of L2 capsid protein (Fig. 1). Finally, transfection of HPV L2 ORF in NIH 3T3 cells resulted in a marked reduction in miR-125b levels. Taken together, these data suggest that hsa-miR-125b may be able to counteract HPV replication and that the virus induces the inactivation of miR-125b. However these conclusions will need to be supported by more direct studies on the human cells naturally infected by HPV.

## 6. Human hepatitis B virus

Human hepatitis B virus (HBV) is a widely spread hepatotropic virus that causes persistent infections [37]. HBV is characterized by a circular DNA genome of 3.2 kb which is only partially double-stranded [38]. This is replicated through an RNA intermediate, the pregenomic RNA, which is produced by transcription of the entire viral DNA by cellular RNA polymerase. Pregenomic RNA is then converted into the viral DNA genome by the HBV polymerase, a multifunctional enzyme with reverse transcriptase, DNA-dependent DNA polymerase, and RNase H activities [39]. The analysis of the hepatitis B virus (HBV) genome by the computer program MiRanda led to the identification of seven sites that are potential targets for human liver microRNAs. These sites were found to be clustered in a 995 bp segment within the viral polymerase ORF and the overlapping surface antigen ORF, and conserved among the most common HBV subtypes. The HBV genomic targets were then subjected to a validation test based on cultured hepatic cells (HepG2, HuH-7, and PLC/PRF/5) and luciferase reporter genes. In this test, one of the selected microRNAs, hsa-miR-125a-5p (Fig. 1), was found to interact with the viral sequence and to suppress the reporter activity markedly. The microRNA was then shown to interfere with the viral translation, down-regulating the expression of the surface antigen [40].

Another study has shown that two other human microRNAs targeting the overlapping surface antigen and polymerase ORFs, hsa-miR-199a-3p and hsa-miR-210, are also able to suppress HBV replication in cultured hepatic cells [41] (Fig. 1).

## 7. Conservation of the microRNA target sequences

Most of the results described above have been obtained in vitro, studying viral replication in cultured mammalian cells. On the other hand there are studies showing that cellular microRNAs can affect viral replication in vivo. One investigation on vesicular stomatitis virus (VSV) infection in mice showed that miR-24 and miR-93 can target viral large protein and phosphoprotein genes and that lack of these miRNAs leads to hypersusceptibility to VSV infection [42]. Another study has confirmed the positive effect of hsa-miR-122 on HCV replication: treatment of chronically infected chimpanzees with a locked nucleic acid-modified antisense inhibitor of miR-122 leads to long-lasting suppression of HCV viremia [43]. These results support the emerging concept that some mammalian microRNAs can directly affect viral gene expression by interacting with viral mRNAs or RNA genomes [44]. This is also supported by computational studies showing that human miRNAs preferentially target the genomes of human-infecting viruses compared to invertebrate viruses [45], whereas plant miRNAs target plant viruses more efficiently than the genomes of animal-infecting viruses [46]. Finally, it is known that several miRNA target sequences are well conserved in viral genomes. To gain further insights into this aspect of microRNA biology, we consulted nucleotide databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). This search revealed that several thousands sequences have been deposited for each of the pathogenic viruses considered in this article. The total number of entries was 35758 for HBV, 110396 for HCV, 369985 for HIV-1 and 5577 for HPV. Sequences belonged to various viral genotypes, subtypes, serotypes, or isolates, and most of them covered only part of the viral genome. Selection of the entries containing the reported miRNA target sites, was performed by Blast search of the miRNA-pairing sequences described in Fig. 1. This search yielded a total number of targets in each viral database ranging from 1754 for hsa-miR-210 to 22199 for hsa-miR-150 (Fig. 2). Further examination of these sequences revealed that many of them are well

| Virus | human miRNA | Total targets in the database[a] | identical targets[b] (%) | conserved targets[c] (%) |
|---|---|---|---|---|
| HBV | 125a-5p | 7652 | 4275 (56) | 6465 (84) |
| | 199a-3p | 20153 | 2767 (14) | 20105 (100) |
| | 210 | 1754 | 1 (0.06) | 4 (0.2) |
| HCV | 196 | 4599 | 15 (0.3) | 258 (6) |
| | 199a-3p | 2913 | 941 (32) | 1440 (49) |
| | 448 | 2898 | 25 (1) | 2559 (88) |
| HIV-1 | 28 | 5568 | 2673 (48) | 5273 (95) |
| | 29a | 22020 | 839 (4) | 14229 (65) |
| | 125b | 20007 | 4405 (22) | 13085 (65) |
| | 150 | 22199 | 10823 (49) | 16090 (72) |
| | 223 | 13247 | 2250 (17) | 10272 (78) |
| | 382 | 5241 | 108 (2) | 4297 (82) |
| HPV | 125b | 2751 | 115 (4) | 962 (35) |

**Fig. 2.** Pathogenic viruses targeted by human microRNAs and conservation of their target sequences. Target sequences were identified by Blast search of nucleotide collections at NCBI using as queries the miRNA-pairing sequences reported in Fig. 1. Search was restricted to selected viruses by using their taxonomy IDs: 10407 for HBV, 11103 for HCV, 11676 for HIV-1, and 333750 for HPV. Target sequences were then downloaded and examined to verify their conservation. [a]Number of viral sequences homologous to the reported microRNA target. [b]Number of viral sequences identical to the reported microRNA target. [c]Number of targets showing a seed-matching sequence identical to the reported one.

conserved. For example over 50% of the HBV targets for hsa-miR-125a are identical to the one reported [40] and 84% display an identical seed-matching sequence (Fig. 2). Other examples of well conserved sequences are given by the HIV-1 targets for hsa-miR-28 and 150, whose seed-matching sites are identical to the reported ones for 95% and 78% of the entries, respectively. Finally, the HCV target for hsa-miR-448 is also fairly conserved.

Given the high mutation rate of viral polymerases, the existence of some well-conserved viral genomic sequences suggests that a selective pressure may be preserving them. This may be due to an essential role that the sequence plays for its (1) protein-coding properties, sometimes with two overlapping open reading frames, (2) gene expression regulatory signals, such as those that control RNA splicing or stability, (3) RNA secondary structure, and/or (4) ability to bind to a host miRNA (see below). In the case of HBV, the mRNA sequence targeted by hsa-miR-125a-5p (Fig. 1) encodes amino acid residues 244–252 of polymerase and 64–72 of surface antigen, with the latter residues falling within the extracellular pre-S1 domain that is responsible for receptor binding on hepatocytes [40].

The available data on the antiviral effects of mammalian microRNAs collected both in vitro and in animal models, the knowledge that some mammalian viruses encode suppressors of RNA-induced silencing, and the theoretical considerations about the conservation of the microRNA target sites, suggest that some human microRNAs effectively contribute to the host defense by targeting essential viral genes, thereby reducing the replication efficiency of the virus. It can be hypothesized that this effect accompanies the well known immune response producing an overall antiviral effect that in some cases would be sufficient to recover from the infection (as for many HBV-infected individuals) whereas in many others would only counteract it, leading to a persistent infection (the other HBV-, HCV-, HIV-1-, and HPV-infected patients). The latter outcome may also be part of the replication strategy of the virus, because it leads to a long spreading ability often lasting one or more decades. If this is the case, then the miRNAs with antiviral effects may be considered host molecules that viruses co-opt to suppress their own replication and to establish a persistent infection, as already proposed [47,48]. Then the miRNA target sequences in viral genomes would be conserved also for their ability to make the virus sensitive to the action of host miRNAs. In this scenario, microRNAs with antiviral effects may be considered fine regulators of virus-host coexistence, leading to persistent infections that are beneficial both for the host survival and the viral spread.

## 8. Concluding remarks

In our opinion, the available experimental data on the role of microRNAs in virus–host interactions sufficiently prove that binding of human microRNAs to viral RNA genomes or transcripts can occur under natural conditions. Furthermore, the knowledge that some target sequences for binding of cellular miRNAs are well conserved in viral genomes supports the concept that such interactions have a biological significance. However, a general conclusion on the role of these interactions cannot be drawn yet. One possibility is that they are beneficial for the host only, with some miRNAs being part of the host innate antiviral defense. Otherwise, the miRNA–RNA interaction may be beneficial for the virus only, as in the case of miRNA-122 acting on HCV. Finally, the interaction may be beneficial for both the host and the virus, as already proposed in the previous paragraph, because it possibly leads to a persistent infection with a long host survival and a high spread of the virus in the human population.

Independently of their biological role, human microRNAs with antiviral effects may be exploited for the development of new strategies for antiviral intervention. Delivery of synthetic miRNA mimics or treatments that modulate the expression of cellular miRNAs could in fact represent useful strategies to unbalance the virus-host coexistence and re-direct the outcome from a persistent infection to a complete recovery. In this regard, priority should be given to those miRNAs whose targets are well-conserved in the viral population. A key impulse in this field may also be given by the study of miRNA gene promoters, eventually leading to the development of treatments that increase their activity in infected tissues. Otherwise, the use of vectors for the ectopic expression of miRNA mimics [49–52] directed towards conserved target sites can also be used to boost the cellular reservoir of antiviral microRNAs.

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